Immunological Evidence for the Presence of Advanced Glycosylation End Products in Atherosclerotic Lesions of Euglycemic Rabbits

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Abstract Atherosclerosis is known to be accelerated in diabetic patients, but the mechanisms of this acceleration are poorly understood. Nonenzymatic glycosylation of long-lived proteins results in the formation of advanced glycosylation end products (AGEs), which are extensively cross-linked and could contribute to atherogenesis. Oxidative modification of LDL is also an important process in atherogenesis. In vitro evidence suggests that hyperglycemia may enhance lipid peroxidation, and conversely, that increased lipid peroxidation may enhance AGE formation. If such interactions occur in vivo, we hypothesized that AGE should be found in atherosclerotic lesions of euglycemic LDL receptor–deficient rabbits in areas rich in lipids and oxidized lipoproteins. To demonstrate the presence of AGEs, we developed antisera against a specific "model" compound of AGE, 2-furoyl-4(5)-(2-furanyl)-*1H*-imidazole (FFI) by using FFI-hexanoic acid (FFI-HA)–protein adducts as the antigen and against AGEs in general by using AGE-albumin as the antigen. Antisera generated with FFI-HA–protein adducts recognized FFI-HA alone as well as FFI-protein adducts. Native proteins or proteins conjugated with aldehydes formed during lipid peroxidation in vitro were not recognized by these antisera. Immunocytochemistry with both FFI-specific and AGE-specific antisera revealed the presence of these epitopes in atherosclerotic lesions of euglycemic LDL receptor–deficient rabbits but not in normal aortic tissues. AGE epitopes within atherosclerotic lesions were predominantly found in similar locations as epitopes generated during modification of the lipoproteins by oxidation, consistent with the hypothesized interactions between oxidation and glycosylation. Indirect evidence in support of the in vivo presence of FFI-like structures was also obtained by the observation that both diabetic and euglycemic human subjects contained autoantibodies that recognize FFI-protein adducts. Taken together, these data provide immunological evidence for the in vivo presence of FFI-like structures and other AGE-protein adducts in atherosclerotic lesions, even in euglycemic conditions. (*Arterioscler Thromb Vasc Biol.* 1995;15:571-582.)

Key Words ● advanced glycosylation end products ● arteriosclerosis ● immunocytochemistry ● oxidation ● autoantibodies

V ascular pathology represents one of the major long-term complications of diabetes mellitus in both the microcirculation and major vessels (in the form of atherosclerosis). Although epidemiological studies have demonstrated that arteriosclerosis is enhanced by diabetes,[1] the mechanisms by which this occurs are not known. Nonenzymatic glycosylation (NEG) of proteins by reducing sugars leads to the formation of advanced glycosylation end products (AGEs).[2-7] By interfering with functionally important lysine groups and amino-terminal amines, AGE formation has the potential to significantly affect biological and structural properties of proteins and has been postulated to contribute to the progression of many diabetic complications.

Both NEG and AGE formation could enhance atherogenesis by several mechanisms. NEG of LDL and other lipoproteins[8,9] could alter lipoprotein metabo-

lism.[10,11] In addition, AGE modification has also recently been demonstrated in circulating lipoproteins.[12] In the vascular wall, formation of AGE adducts may interfere with vasodilation either by the reduced elasticity of AGE-modified structural proteins[2,3] or by interference of AGEs with the vascular response to nitric oxide.[13] AGE formation may trap LDL or immunoglobulins in the intima by cross-linking to structural proteins.[14,15] In turn, the prolonged intimal residence time of LDL may enhance the generation of oxidized LDL (OxLDL), which may contribute to atherogenesis by multiple mechanisms,[16-18] including rapid uptake of OxLDL via scavenger receptors.[19] Macrophages also possess specific receptors that recognize AGE proteins,[20-24] and uptake of AGE-modified lipoproteins via these receptors could contribute to foam cell formation. AGEs could also enhance the intimal accumulation of macrophages either by binding to specific receptors on endothelial cells[24] or by a direct chemotactic effect[25,26] similar to that of OxLDL.[27] Finally, by binding to one of the AGE receptors, AGE-modified proteins may induce endothelial cells to generate a pro-oxidant state.[28]

A number of in vitro studies suggest that lipid peroxidation and NEG/AGE formation involve some similar intermediates. In vitro, hyperglycemia enhances lipid peroxidation, and conversely, increased lipid peroxidation could augment AGE formation.[12,29-35] If this mutual
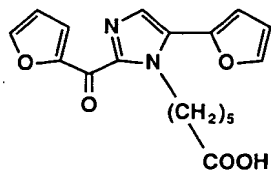
FIG 1.   Structure of 2-(2-furoyl)-4(5)-(2-furanyl)-*1H*-imidazole–hexanoic acid (FFI-HA).

enhancement also takes place in vivo, it would imply that accelerated formation of AGE products should occur in atherosclerotic lesions of diabetic subjects. Indeed, enhanced lipid peroxidation in general[36,37] and AGE formation in particular[12] have been demonstrated in circulating lipoproteins of diabetic subjects, and AGE formation in atherosclerotic tissues of diabetics has been demonstrated by immunocytochemistry.[38] However, it is not possible to estimate if and how much enhanced lipid peroxidation contributes to increased AGE formation in diabetic subjects, as AGE formation may simply be a direct consequence of hyperglycemia. To address this question, we hypothesized that enhanced AGE formation may also occur in euglycemic Watanabe heritable hyperlipidemic (WHHL) rabbits, which are characterized by extreme hypercholesterolemia. The atherosclerotic lesions of these rabbits are rich in OxLDL,[39-42] and antioxidant therapy can inhibit atherosclerosis in this animal model.[16,17] Therefore, the extensive lipid peroxidation that occurs in their lesions should enhance AGE formation, and their atherosclerotic lesions should contain significant amounts of AGEs in contrast to normal rabbit aortas.

To test the hypothesis that products of AGEs would be formed in atherosclerotic lesions of euglycemic WHHL rabbits, we developed antisera against a specific compound of AGE, 2-furoyl-4(5)-(2-furanyl)-*1H*-imidazole-1–hexanoic acid (FFI), by using FFI–hexanoic acid (FFI-HA) (Fig 1) as the antigen, as well as antisera against AGEs in general, using AGE-albumin as the antigen. The present article characterizes the resulting antisera and provides immunocytochemical evidence for the occurrence of FFI-like epitopes and other AGE epitopes in atherosclerotic lesions of euglycemic WHHL rabbits. Furthermore, we show that IgA, IgG, and IgM autoantibodies that recognize FFI-protein adducts are present in the sera of human diabetic and control subjects, thereby providing additional evidence for the formation of FFI-like epitopes in vivo.

Methods

Isolation of LDL

LDL (1.025 to 1.070 g/mL) was prepared from male guinea pigs by sequential ultracentrifugation, as previously described.[43] The isolated lipoprotein was extensively dialyzed against phosphate-buffered saline (PBS; 0.15 mol/L NaCl, 36 mmol/L phosphate buffer, pH 7.35) containing 0.27 mmol/L EDTA. The protein content was determined by the method of Lowry et al,[44] and LDL was sterile-filtered for further use.

Preparation of Immunogens and Competitors for Radioimmunoassays (RIAs)

Conjugation of FFI-HA to proteins was performed with a modification of the method described by Gendloff et al.[45] FFI-HA was kindly provided by Dr Michael Yamin. FFI-HA

was conjugated to guinea pig LDL, guinea pig albumin, or keyhole limpet hemocyanin (KLH) as follows. FFI-HA (1 mg, or 2.92 μmol) was added to equimolar amounts of *N,N'*-dicyclohexylcarbodiimide (Pierce Chemical Co) and *N*-hydroxysuccinimide (Pierce) in 0.1 mL tetrahydrofuran (Pierce) and gently mixed for 24 hours at room temperature. *N*-hydroxysuccinimide enhances the coupling reaction between the hapten and the protein mediated by the water-soluble carbodiimide. Precipitates were removed by filtration through a 0.45-μm filter. The reaction mixture was washed with tetrahydrofuran, dried under nitrogen, and resolubilized in 200 μL dimethylformamide per milligram of FFI. Before use, LDL was extensively dialyzed against 0.1 mol/L NaHCO₃, pH 7.35, containing 10 mg/mL EDTA (to avoid oxidative modification during the procedure) and adjusted to 5 mg in 0.5 mL. The reaction mixture containing FFI-HA was added dropwise to LDL under constant stirring (up to a total of 200 μL or until excessive aggregation was observed) and allowed to react for 24 hours in a light-proof vial. The conjugate was then extensively dialyzed, first against 0.1 mol/L NaHCO₃ with EDTA and then against PBS, pH 7.35, also containing 10 mg/mL EDTA. The product of this reaction was termed FFI-LDL. Conjugation of FFI to guinea pig albumin or KLH was performed similarly, also at a ratio of 1 mg FFI-HA per milligram of protein, and yielded FFI-albumin and FFI-KLH, respectively.

AGE-modified proteins were also prepared according to the method of Stern and colleagues (Schmidt et al[26]). LDL, albumin, hemoglobin, and other proteins were incubated with either 500 mmol/L glucose, 500 mmol/L glucose-6-phosphate, or 250 mmol/L ribose in the presence of aprotinin (2 μg/mL), leupeptin (0.5 μg/mL), pepstatin (0.7 μg/mL), PMSF (1.5 mmol/L), EDTA (1 mmol/L), and BHT (1 mg/mL). Incubation of sterile-filtered solutions was carried out for 3 months at 37°C. Solutions were then extensively dialyzed against PBS and stored at 4°C. Malondialdehyde (MDA)-LDL and copper-oxidized LDL (Cu²⁺-LDL) were prepared as described.[43]

Immunization Procedures

Guinea pigs were immunized with FFI-HA conjugated to guinea pig LDL or albumin. The rationale for the choice of these proteins was the observation that homologous LDL is a powerful vehicle for presenting an immunogen and results in the formation of antisera specific for the modified lysine residues only; ie, the antibodies recognize modified lysine on a number of different proteins.[46] Homologous protein was used to avoid the generation of antibodies against epitopes of the native protein. In addition, we also immunized guinea pigs with FFI-HA conjugated to KLH. Antibodies against epitopes of KLH, the carrier protein, should not recognize mammalian proteins. Finally, we immunized guinea pigs with AGE-albumin. Two guinea pig antisera were generated for each antigen. The primary immunization usually consisted of 150 μg protein/0.5 mL PBS in Freund's complete adjuvant, and booster immunizations were performed biweekly with 100 μg protein in Freund's incomplete adjuvant, as previously described.[43] Antibody titers were determined in the preimmune sera and 5 to 7 days after the second and subsequent boosts.

Determination of Antibody Titers and Specificity

Titers and specificity of both induced antibodies and autoantibodies were determined by solid-phase RIA techniques, as previously described.[43,46] Antigen was coated at 5 μg/mL for 2 hours at 37°C. The amount of bound immunoglobulin was quantitated with a radiolabeled secondary antibody; eg, guinea pig IgG was detected by goat anti–guinea pig IgG (Organon-Teknika–Cappel) labeled at ≈10 000 cpm/ng with ¹²⁵I, using lactoperoxidase (Enzymobeads, Bio-Rad Laboratories). The plates were incubated for 4 hours at 4°C with ≈400 000 cpm per well of the secondary antibody. Results were expressed as antibody binding as a function of antibody dilution. Titers were defined as the reciprocal of the highest dilution that gave

binding values exceeding three times that of the preimmune control. Competitive solid-phase RIAs were performed similarly except that the antigen was plated at 1 or 2 μg/mL. A limiting and fixed dilution of the primary antibody (25 μL) was then added together with an equal volume of dilution buffer (3% bovine serum albumin, 0.02% NaN_3, 0.05% Tween 20, and 0.001% aprotinin in PBS) containing increasing amounts of potential competitors. The results were calculated as B/B_0, ie, the amount of antibody bound to the plated antigen in the presence of competitor (B) divided by the binding in the absence of competitor (B_0).

Competitive solid-phase RIAs were used to test the ability of antibodies that were generated against FFI-protein adducts to recognize epitopes on "physiologically" formed AGEs. AGEs were initially generated by incubation of LDL or albumin with 100 mmol/L glucose in the presence of EDTA under sterile conditions, in the dark, at 37°C for periods of 2 to 9 months. Aliquots of these preparations were also subjected to proteinase K digestion, acid hydrolysis, or acid hydrolysis and subsequent reaction with ammonia.[47] Enzymatic digestion was carried out by adding proteinase K (Sigma Chemical Co) to LDL or albumin at a protein to enzyme ratio of 10:1 and incubating the resulting mixture at 37°C for 40 hours. Proteinase digestion was stopped by addition of 0.1 mol/L PMSF (Sigma) in ethanol to a final concentration of 1 mmol/L. Acid hydrolysis was performed by incubating the proteins with 6N HCl for 16 hours at 110°C. Samples were then dried under nitrogen, washed four times with distilled water, and resuspended in PBS.

Determination of Autoantibodies

The sera of 40 well-characterized diabetic patients (19 men and 21 women; age range, 19 to 82 years; mean age, 37 years; type of diabetes, 12 type I and 28 type II; range of duration of diabetes, 0.2 to 35 years; mean duration, 12.2 years; HbA1c, $8.7\pm2.0\%$) and 40 age- and sex-matched euglycemic control subjects (17 men and 23 women; age range, 16 to 80 years; mean age, 39 years; HbA1c, $5.2\pm0.4\%$) were tested for autoantibodies to FFI-LDL, plated as the antigen in solid-phase RIAs. A fixed dilution of the sera was used as the primary antibody (1:8 dilution for IgA and IgG and 1:256 dilution for IgM autoantibodies), and the amount of autoantibody bound to the plated antigen was determined by using radiolabeled monoclonal anti-human IgM, IgG, or IgA (Zymed) as the second antibody. Results of these assays were expressed as counts of second antibody bound to the human autoantibodies. For each serum sample, a control value was subtracted, which was determined in a duplicate set of wells without a specific antigen. Selected sera from both control and diabetic subjects were also subjected to competitive RIA to determine the specificity of antibody binding to FFI-LDL. Student's *t* test was used to compare autoantibody concentrations between diabetic and control subjects, and ANOVA was used to test for correlations between antibody concentrations and clinical parameters.

Tissue Preparation and Immunocytochemistry

Aortas of WHHL rabbits were perfusion-fixed at physiological pressure with formal sucrose (4% paraformaldehyde, 5% sucrose, 20 mmol/L BHT, and 1 μmol/L EDTA, pH 7.4) as described.[48] After dissection, the aortas were subjected to immersion-fixation for an additional 12 hours. Aortas of control and 28-week alloxan diabetic Lewis rats were flash-frozen in liquid nitrogen; portions of these samples were subsequently fixed in paraformaldehyde and embedded in paraffin.

Serial 8-μm-thick sections on microscope slides were rehydrated and immunostained with an avidin/biotin/alkaline phosphatase system (Vector Labs) as previously described.[48,49] The primary antisera against epitopes of AGEs that were used are listed in the Table. In addition, some sections were stained with the respective preimmune sera. Arterial sections of atherosclerotic WHHL aortas were also stained with antisera and mono-

Guinea Pig Antisera Against Presumed Epitopes of Advanced Glycosylation End Products (AGEs)

Antiserum	Immunogen	Titer
FL-1	FFI-LDL (gp)	100 000
FA-1	FFI-albumin (gp)	100 000
FK-1	FFI-KLH	100 000
GPA-1	AGE-albumin (gp)	100 000

FFI indicates 2-furoyl-4(5)-(2-furanyl)-*1H*-imidazole; KLH, keyhole limpet hemocyanin; and gp, guinea pig. Antisera from two different guinea pigs were generated against each of the FFI-modified proteins and against AGE-albumin. Shown are the titers of the specific antiserum from each immunogen class used for immunocytochemistry, but similar results were obtained with other antisera. Titers indicate binding to the respective immunogen.

clonal antibodies against epitopes generated during the oxidative modification of LDL, ie, MDA2 (specific for MDA-lysine) and NA59 (specific for 4-hydroxynonenal–lysine).[40,43] Controls included MB47, a monoclonal antibody against native apo B-100[50]; RAM-11, a macrophage-specific monoclonal antibody[51]; and nonspecific sera. Competitive immunostaining was performed by preincubating the antibody with excess potential competitor for 1 hour before immunostaining and by comparing the resultant staining to that obtained with the same final dilution of antibody in the absence of competitor. To rule out nonspecific binding to the competitor, controls included comparative immunostaining with a nonspecific antibody preincubated with the same competitor (eg, staining with RAM-11, a macrophage-specific monoclonal antibody, should not be affected by preincubation with AGE-LDL).

Results

Characterization of Antisera to FFI-Protein Adducts

Even minor modification of lysine residues renders LDL highly immunogenic,[46] and previous studies from this laboratory have shown that immunization of guinea pigs with modified homologous LDL and albumin re-
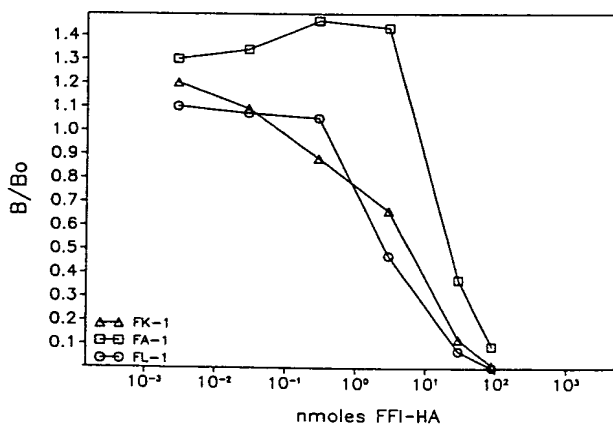

FIG 2. Solid-phase competitive radioimmunoassay (semilog) demonstrating the specificity of antisera FL-1, FA-1, and FK-1. Wells of microtiter plates were coated with either FFI-LDL (for FL-1), FFI-albumin (for FA-1), or FFI-keyhole limpet hemocyanin (KLH) (for FK-1), as described in "Methods." A fixed and limiting dilution of FL-1 (1:10 000 dilution), FA-1 (1:10 000 dilution), or FK-1 (1:25 000 dilution) was added to the respective plates in the absence or presence of increasing amounts of FFI-HA. Results are expressed as B/B_0, where B is the binding of the antiserum to the plated antigen in the presence of competitor and B_0 the binding in the absence of competitor. Other abbreviations as in the legend to Fig 1.
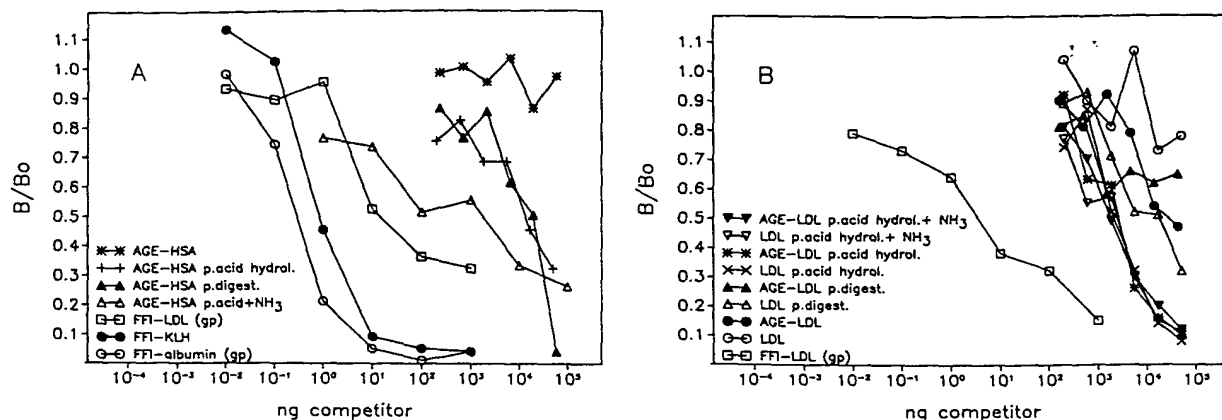
FIG 3. Two solid-phase competitive radioimmunoassays (semilog) of guinea pig antiserum FL-1 generated by immunization with homologous FFI-LDL. Human FFI-LDL (1 mg/mL) was plated as the antigen. FL-1 at a dilution of 1:10 000 (A) or 1:20 000 (B) was added together with increasing concentrations of indicated competitors. Antibodies that bound to the plated antigen were detected with ^{125}I-labeled goat anti–guinea pig IgG. Results are expressed as B/B_0, where B is the binding of FL-1 in the presence of competitor and B_0 binding in the absence of competitor. LDL p. digest. indicates LDL subjected to proteinase K digestion as described in "Methods"; AGE, advanced glycosylation end products; HSA, human serum albumin; p. digest., proteinase K digestion; acid hydrol., acid hydrolysis; NH_3, treatment with ammonia; and KLH, keyhole limpet hemocyanin. Other abbreviations as in the legend to Fig 1.

sulted in monospecific antisera that recognized the adduct formed but not the native protein.[52] In the present experiments, we wanted to generate antisera that would recognize FFI conjugated to proteins via lysines, as such FFI-lysine conjugates have been hypothesized to represent one of the structures generated during AGE formation. We used FFI-HA (Fig 1) as a model of FFI conjugated to lysine[53] and covalently linked this to lysine residues of LDL by using a modification of the carbodiimide method described by Gendloff et al,[45] which directly links the carboxy-terminal carbon of HA to the ε-amino group of lysine. Thus, this coupling reaction does not generate any intermediary "linker" between the hapten and carrier, as occurs when haptens are linked via the commonly used glutaraldehyde method. (In fact, in preliminary experiments with glutaraldehyde as the linker, we generated antisera that contained a population of antibodies that recog-

nized glutaraldehyde-modified LDL.) It should be kept in mind, though, that FFI-HA itself models FFI-lysine. Immunizations of guinea pigs with FFI-HA conjugated with homologous LDL (n=2), homologous albumin (n=2), and KLH (n=2) yielded high titers of antibodies that bound to both FFI-LDL and the respective antigen (Table). These antisera showed no significant antibody titer against native, unmodified LDL or albumin.

To determine the specificity of each antiserum, we performed classic competition RIAs. In these assays, the various immunogens (ie, FFI-LDL, FFI-albumin, or FFI-KLH) were plated as the solid-phase reactant in wells of microtiter plates, and fixed and limiting dilutions of antisera were added in the absence or presence of increasing concentrations of FFI-HA or FFI-modified proteins. In these assays, equal amounts (by mass) of the modified proteins were added because we have no accurate method to measure their absolute FFI-lysine
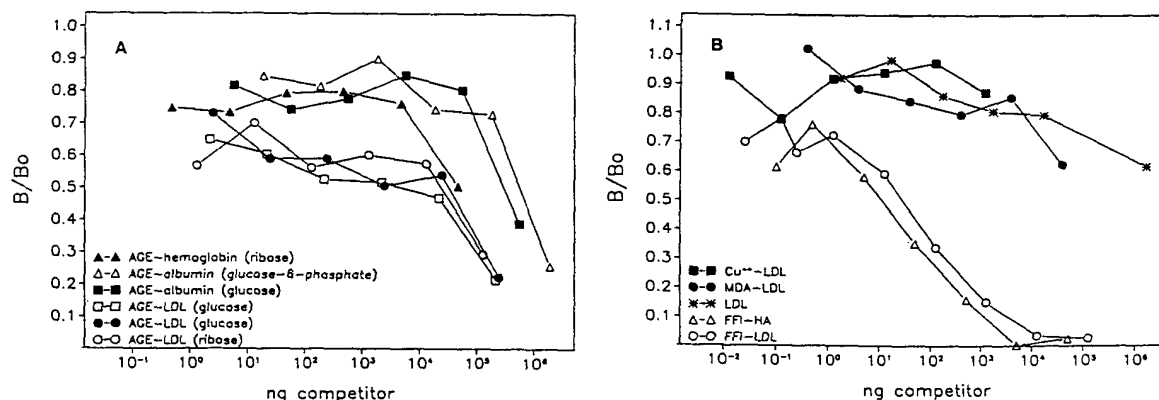

FIG 4. Further characterization of FL-1, the antiserum generated with FFI-LDL, by competitive radioimmunoassay (semilog). FFI-LDL was plated as the antigen (2 μg/mL), and a 1:50 000 dilution of FL-1 was added together with increasing concentrations of competitors as described in the legend to Fig 2. A, Competition by a number of AGE-protein preparations generated by long-term incubation with different sugars, as described in "Methods." The sugar used to generate each preparation is indicated in parentheses. B, Malondialdehyde-modified LDL, Cu^{2+}-oxidized (Ox) LDL, or native LDL used to generate these forms of OxLDL compete very poorly and only at high concentrations. HA indicates hexanoic acid. See the legend to Fig 3 for explanation of other abbreviations.
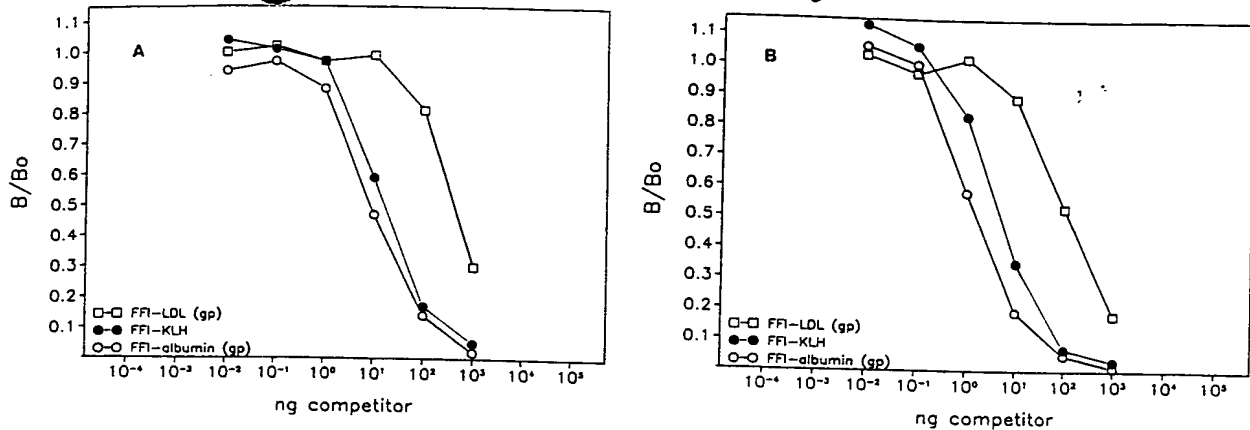
FIG 5. A, Competitive radioimmunoassay (RIA) (semilog) of guinea pig antiserum FA-1 generated by immunization with homologous FFI-albumin. FFI-albumin (1 µg/mL) was plated as the antigen. FA-1 at a dilution of 1:10 000 was added together with increasing concentrations of competitors. Antibodies that bound to the plated antigen were detected and results were expressed as described in the legend to Fig 2. B, Competitive RIA of antiserum FK-1, generated by immunization with FFI conjugated to KLH, using FFI-KLH (1 µg/mL) as the antigen and a 1:25 000 dilution of FK-1. See the legend to Fig 3 for explanation of other abbreviations.

content. Thus, the assays give evidence of the specificity (ability to compete) and of the relative affinity (degree of parallelism of the slopes of the competition curves) of the antisera but do not allow quantitative comparisons between competitors, as the modified proteins contain different absolute numbers of FFI-lysine adducts.

As shown in Fig 2, FFI-HA was able to completely compete for the binding of each antiserum to its respective immunogen. In other words, FFI-HA completely prevented the binding of antiserum FL-1 to FFI-LDL or of FA-1 to FFI-albumin. Native LDL, when subjected to a "mock" conjugation (ie, when exposed to the same conditions used to prepare FFI-LDL but in the absence of FFI-HA), did not compete (data not shown). Furthermore, neither OxLDL nor MDA-LDL competed for binding to antiserum FL-1. Together, these data clearly demonstrate that the antisera recognize FFI-HA alone
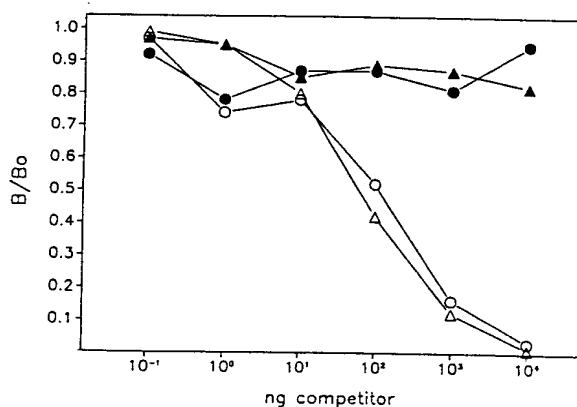

FIG 6. Competitive radioimmunoassay (semilog) with two antisera (GPA-1 and GPA-2) generated against guinea pig AGE-albumin. AGE-albumin was plated as the antigen (2 µg/mL), and a 1:2000 dilution of each antiserum was added together with increasing amounts of competitor. Results are expressed as B/B₀. Binding of both antisera to the plated antigen was effectively competed by AGE-albumin (open symbols), whereas FFI-LDL did not compete (closed symbols). See the legend to Fig 3 for explanation of other abbreviations.

as well as FFI-HA conjugated to the carrier protein used for immunization.

To document that each antiserum would also recognize FFI-HA when conjugated to other proteins, additional competitive assays were performed. Fig 3 shows recognition of various glycosylated and nonglycosylated proteins by guinea pig antiserum FL-1, which was generated by immunization with homologous FFI-LDL. Clearly, this antiserum recognized FFI-protein adducts, irrespective of the protein to which the FFI-HA was bound, ie, FFI-LDL, FFI-KLH, or FFI-albumin (Fig 3A). Although exact quantitation cannot be extracted from these data (as noted above), it nevertheless appears that the FFI present on albumin was "seen" better by the antisera than was the FFI present on LDL. This could be due to the fact that many of the modified lysine residues on FFI-LDL were located at sites inaccessible to the antibody or that were sterically hindered, ie, by lipid. In contrast, modified lysine sites on albumin, a much smaller and simpler protein, are more likely to be readily available in solution to the antibodies. The fact that the competition curves are, in general, parallel implies that those FFI-lysine residues available to the antibodies are recognized with approximately equal affinity.

Although FFI was identified as an AGE,[53,54] the natural occurrence of FFI has been questioned, as it may be formed during extraction and purification procedures as a by-product of acid hydrolysis of glycated proteins in the presence of ammonia.[47,55-57] We therefore tested whether the antibodies generated against FFI-LDL would also recognize more generalized AGE preparations generated by long-term incubations of LDL or serum albumin with glucose. The ability of the FFI-specific antisera to recognize these AGE proteins showed considerable variability: AGE–human serum albumin (AGE-HSA) was not usually recognized (Fig 3A). Some AGE-LDL preparations were recognized to a modest extent, albeit only when added in very high concentrations (Fig 3B). However, when AGE-LDL or AGE-HSA was subjected to proteinase digestion or acid hydrolysis (in the absence of ammonia), there was increased recognition of the resulting breakdown prod-
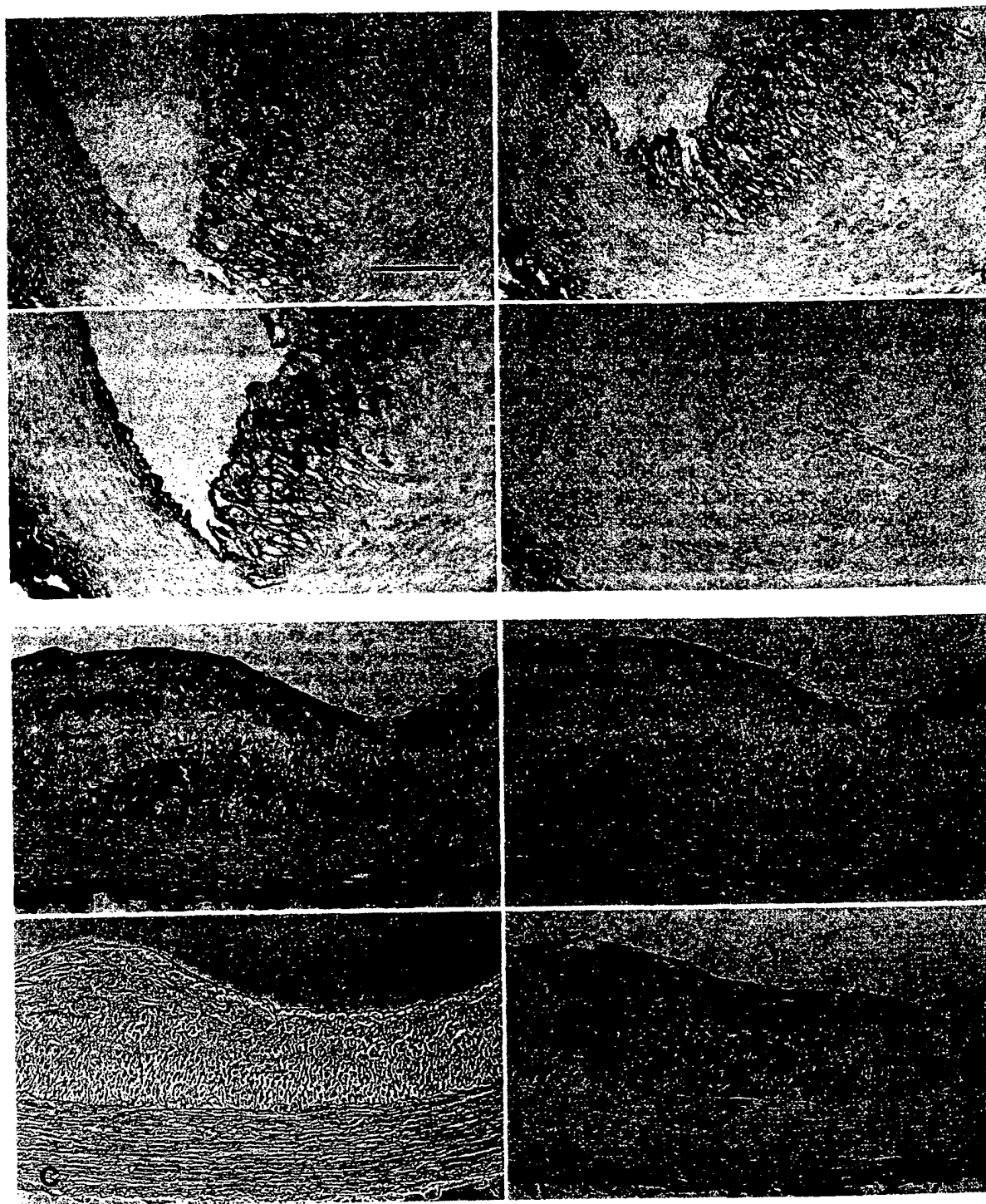
ucts. Presumably, the latter treatments expose epitopes otherwise sterically hindered from interacting with the antibodies. The reaction with ammonia appeared to increase immune recognition of some AGE-HSA preparations reported as previously by Horiuchi et al[47] but did not enhance immune recognition of the initial FFI-LDL preparations (Fig 3B). By contrast, several AGE proteins prepared with extremely high concentrations of glucose, glucose-6-phosphate, or ribose (250 to 500 mmol/L; see "Methods") contained epitopes recognized by FL-1 (Fig 4A). This demonstrates that with very high concentrations of sugars, sufficient FFI-lysine adducts were formed to enable detection by FL-1, FA-1, and FK-1, even without acid hydrolysis or protein digestion.

Competition assays with FL-1 also demonstrated that the epitopes formed during oxidative modification of LDL

Fig 7. Comparative immunocytochemical staining of a transitional lesion from a Watanabe heritable hyperlipidemic rabbit aorta with antisera specific for FFI-lysine epitopes. Lesions were stained with the avidin/biotin/alkaline phosphatase method, and the presence of the antigen recognized by the primary antibody is indicated by a red substrate. Nuclei were counterstained with methyl green. A, Section stained with a 1:250 dilution of FL-1 (generated with FFI-LDL). B, Section stained with a 1:500 dilution of FA-1 (generated with FFI-albumin). C, Section stained with a 1:250 dilution of FK-1 (generated with FFI-keyhole limpet hemocyanin). D, Competitive immunostain with FL-1 in the presence of excess FFI-albumin. FFI-albumin effectively competed with FFI epitopes in the tissue section for antibody binding (original magnification ×139, bar=100 μm). See the legend to Fig 3 for explanation of other abbreviations.

Fig 8. Immunocytochemical evidence indicating that the epitopes recognized by antisera FL-1 and GPA-1 are AGE specific. A and B, Competitive immunostaining of an advanced atherosclerotic lesion with FL-1, the antiserum generated with FFI-LDL. Section shown in A was stained with a 1:350 dilution of FL-1. Section in B was stained in the same assay and under identical conditions but with FL-1 (400 μL of a 1:350 dilution) that had been preincubated for 1 hour with 1.28 mg human AGE-LDL prepared as described in "Methods." Preincubation of non-AGE-specific antibodies such as RAM-11, the macrophage-specific monoclonal antibody, with the same competitor (AGE-LDL) did not significantly reduce the staining of macrophages in serial sections of the same lesion (data not shown). C, Staining with a 1:250 dilution of preimmune serum from the guinea pig used to generate GPA-1 (phase-contrast view). D, Immunostaining of the same lesion with a 1:250 dilution of GPA-1 (original magnification ×87, bar=100 μm). See the legend to Fig 3 for explanation of other abbreviations.

were not recognized by FL-1. As shown in Fig 4B, MDA-LDL and Cu^{2+}-LDL competed only minimally when added at high concentration, and competition observed with these forms of OxLDL was not significantly greater than that seen with native LDL.

The specificities of the antisera generated with FFI-albumin (FA-1) or with FFI-KLH (FK-1) were similar to that of FL-1. These antisera recognized not only FFI-HA alone (Fig 2) and the respective immunogens but also other FFI-protein adducts (Fig 5A and 5B).

Characterization of Antisera to AGE-Albumin

Immunization with AGE-albumin induced the generation of high-titer antisera (GPA-1 and GPA-2), which recognized AGE-albumin. However, FFI-LDL did not compete with the antisera for binding to AGE-albumin (Fig 6). Thus, neither antiserum cross-reacted with the FFI-lysine epitope.

Immunocytochemistry

To further address the question of the natural occurrence of FFI or an FFI-like epitope, we used the three antisera generated against FFI-LDL, FFI-albumin, and FFI-KLH for immunocytochemical analysis of aortic atherosclerotic lesions of euglycemic WHHL rabbits (mean±SD nonfasting blood glucose, 100±9.4 mg/dL, n=14). Fig 7 shows serial sections of a transitional atherosclerotic lesion stained with FL-1 (A), FA-1 (B), and FK-1 (C). All three antisera recognized FFI-lysine epitopes in almost identical distribution. By contrast, nonlesioned aortic segments showed only very light and diffuse medial staining (data not shown). Significant adventitial staining was found in all sections studied.

Competition studies confirmed the specificity of staining for FFI-lysine adducts: Fig 7D shows a serial section stained with FL-1 that had been preincubated with excess FFI-albumin. Specific staining within the atherosclerotic lesion was virtually abolished. Preincubation of FL-1 with native LDL or MDA-LDL did not affect staining (data not shown). Competitive immunocytochemistry was also used to demonstrate that the epitope recognized by FL-1 is indeed an AGE epitope. Preincubation of FL-1 with AGE-LDL prepared by prolonged incubation of LDL with a high concentration of glucose markedly reduced the staining intensity (Fig 8B) compared with the no-competitor control (Fig 8A). Similar results were also obtained with AGE-LDL prepared with ribose and with AGE-albumin. By contrast, preincubation of FL-1 with fresh LDL and glucose or with MDA-LDL showed no significant competition. Control staining with RAM-11, a monoclonal antibody against macrophages, was not affected by preincubation with AGE-LDL (data not shown).

GPA-1, an antiserum raised against AGE-albumin, also recognized specific epitopes in atherosclerotic lesions (Fig 8D) and yielded staining patterns very similar to those of FFI-lysine–specific antisera. To exclude the possibility that some GPA-1 staining might result from low-titer autoantibodies to MDA-lysine in the sera of many species[17] or from nonspecific binding, serial sections were stained with an identical dilution of preimmune serum from the same animal. The section stained with preimmune serum was devoid of specific staining (Fig 8C).
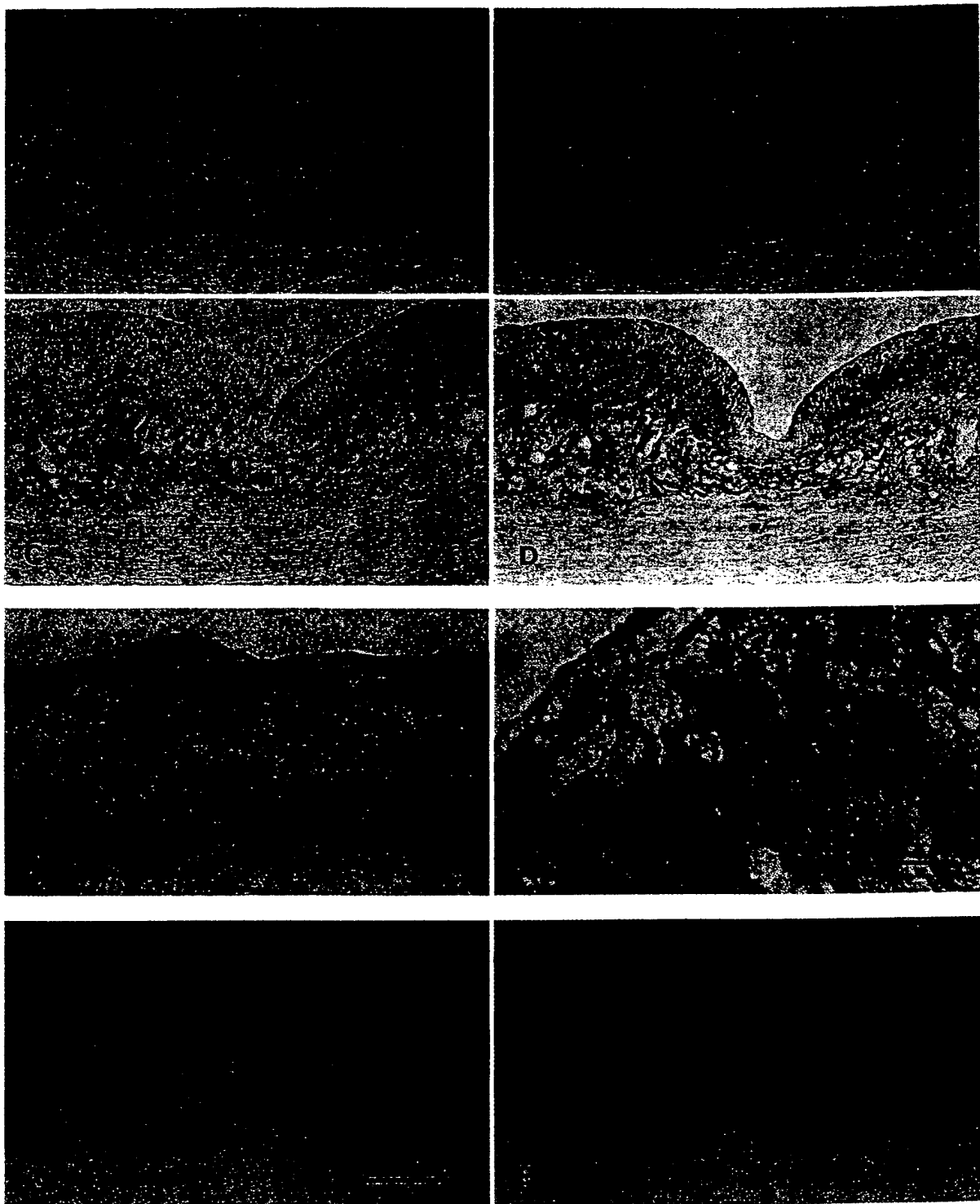
The staining pattern obtained with the different antisera to AGE epitopes was remarkably similar to that obtained with antibodies against epitopes generated during the oxidative modification of lipoproteins (Fig 9). Panels A and D show an advanced lesion from a WHHL rabbit aorta stained with two monoclonal antibodies against "oxidation-specific" epitopes: NA59 (specific for 4-hydroxynonenal–lysine) and MDA2 (specific for MDA-lysine), respectively. Panels B and C show sections that were stained with antisera against AGE epitopes (FL-1 and GPA-1, respectively). At higher magnification (Fig 10A and 10B), the FFI-specific antibodies displayed a more extracellular and diffuse distribution pattern and some staining of the media, as opposed to the more intense macrophage-associated staining often observed in early atherosclerotic lesions with oxidation-specific antibodies. Nevertheless, some intimal macrophages showed intense staining with FL-1 (Fig 10A).

To determine whether qualitative differences in AGE staining could be detected by our antibodies in tissues from a diabetic animal, we also immunostained aortas from diabetic and control rats. Fig 11 shows the thoracic aorta from a control (A) and a diabetic (B) rat stained with a 1:250 dilution of the antibody against FFI-LDL. None of these sections displayed any intimal thickening or other atherosclerotic lesions. Although the immunostaining procedure provides only semiquantitative results, the more extensive and intense staining of the diabetic specimen is apparent, particularly in the entire media. These results were representative of multiple sections examined from the aortas of two diabetic and four euglycemic rats.

Autoantibodies to FFI in the Sera of Human Subjects

Nakamura et al[38] demonstrated the presence of AGE epitopes in cardiac tissue of diabetic subjects. Using our

antisera against FFI-like epitopes, we demonstrated the presence of these epitopes in aortic and renal tissues of both euglycemic and diabetic human subjects (data not shown). Because homologous modified LDL and albumin were immunogenic in guinea pigs and because FFI-like epitopes were observed in human tissues, we determined whether autoantibodies that recognize FFI epitopes were present in the sera of 40 well-characterized diabetic patients and 40 matched euglycemic control subjects. The majority of these serum samples had autoantibody titers (Fig 12). No differences were found between diabetic and control sera in the titers of IgG and IgM autoantibodies, but the diabetic sera had significantly more IgA autoantibodies than did controls

FIG 9. Comparative immunostaining of an advanced atherosclerotic lesion of a Watanabe heritable hyperlipidemic rabbit with antibodies against epitopes formed during oxidative modification of LDL and AGE-specific epitopes. A, Immunostaining with NA59, a monoclonal antibody specific for 4-hydroxynonenal–lysine (dilution 1:200). B, Staining with FL-1, the antiserum generated with FFI-LDL (dilution 1:350). C, Staining with GPA-1, guinea pig antiserum generated with AGE–guinea pig albumin (dilution 1:250). D, Staining with MDA2, a monoclonal antibody specific for malondialdehyde-lysine (dilution 1:400). Note that the distribution of oxidation-specific and AGE-specific epitopes is very similar, although the respective antibodies do not cross-react (original magnification ×111, bar=100 μm). See the legend to Fig 3 for explanation of other abbreviations.

FIG 10. A and B, Details of macrophage-rich areas of advanced lesions stained with FL-1. Note the presence of intense staining in both the extracellular matrix and some macrophage/foam cells (A, original magnification ×279, bar=100 μm; B, original magnification ×696, bar=20 μm).

FIG 11. Comparative immunostaining of aortas from a control rat (A) and a rat after 28 weeks of alloxan-induced diabetes (B) using a 1:250 dilution of FA-1. The media of the diabetic rat stained more intensely than control (original magnification ×139, bar=100 μm).

($P<.0001$). Competitive RIAs were performed with selected sera from both diabetic and control subjects to show the specificity of autoantibody binding to FFI-LDL. FFI-LDL, added as a fluid-phase competitor, was able to compete for autoantibody binding to an extent of 50% to 100% (data not shown). Native LDL or mock-modified LDL (see above) did not compete. In this limited study, IgA autoantibody concentrations of diabetic subjects were not correlated with the type of diabetes, duration of diabetes, or treatment. These data provide additional evidence that FFI-like epitopes exist in vivo in human subjects.

Discussion

Several studies reported the presence of AGE-modified proteins in the circulation.[12,58,59] Increased AGE concentrations were described in tissues from diabetic subjects,[59-62] and Nakamura and colleagues[38] recently used an AGE-specific antiserum to show that AGEs occur in coronary atheromas of some human diabetic subjects. Because lipid peroxidation is thought to be an important component of the atherogenic process and because it could enhance AGE formation, we sought to determine whether AGE products could be detected in atherosclerotic lesions of euglycemic WHHL rabbits.

The structure of individual AGE products and their occurrence in vivo remain largely unknown. Proposed AGE components isolated from AGE proteins generated in vivo include FFI,[53,54] pentosidine,[61] and pyrroles.[62] To document the presence of AGE structures, we generated several monospecific antisera against one model AGE compound, FFI, as well as antisera against AGE-albumin. Using all of these antisera for immunocytochemistry, we demonstrated the presence of AGE products and specifically that of FFI-like structures in atherosclerotic lesions of euglycemic rabbits.

There is controversy in the literature regarding the in vivo existence of FFI as part of the AGE products. Some investigators reported evidence in support of its presence,[53,54] whereas other data have suggested that FFI is formed ex vivo as a result of acid hydrolysis in the presence of ammonia.[55-57] Our antisera recognized FFI-lysine or FFI-like epitopes on a number of untreated AGE preparations obtained by incubation of proteins with high concentration of sugars (Fig 4A). Other AGE proteins prepared with a lower concentration of glucose were recognized only after hydrolysis or proteinase K digestion (Fig 3). This could mean that some FFI-like epitopes are present in intact proteins but are masked and are exposed only after protein hydrolysis or enzyme digestion.[12,58,59] We also found that ammonia treatment of acid hydrolysates of proteins increased immunorecognition by FFI antisera, as suggested by Horiuchi et al.[47] Moreover, epitopes could also be exposed by proteinase K digestion, which should not alter chemical structure in the same way. Makita et al[58,59] also found that similar digestion was needed to maximize epitope exposure. Furthermore, the demonstration of FFI-like epitopes in some untreated AGE proteins and the immunocytochemical demonstration of FFI or FFI-lysine epitopes in atherosclerotic tissues or diabetic aortas not subjected to any interventions clearly indicate the presence in vivo of FFI or FFI-like structures. Immunoreactivity observed in arterial tissues from WHHL rabbits showed a particular abundance of these presumed AGE epitopes within atherosclerotic lesions. Competition studies with FFI and FFI-modified proteins demonstrated the specificity of the immunocytochemical identification of FFI-like epitopes. The ability of AGE-modified proteins to inhibit binding of FFI antisera to tissue sections (Figs 7 and 8) showed that FFI itself or an immunologically closely related epitope is formed during long-term incubation of proteins with various reducing sugars; ie, FFI is a component of AGE.

GPA-1, an antiserum generated against AGE-albumin that did not cross-react with FFI-modified proteins, also recognized epitopes in atherosclerotic lesions (Figs 8D and 9C) and showed staining patterns similar to those obtained with FFI-specific antisera. Together, these data demonstrate that AGE-specific epitopes are formed in atherosclerotic lesions of euglycemic rabbits and that FFI (or structures that are immunochemically very closely related to FFI) is one of these compounds.

When the aortas of 28-week diabetic rats were compared with controls, a more intense staining with the FFI-lysine–specific antibodies was observed in some diabetic aortas (Fig 11). Although immunostaining is only semiquantitative, these findings would agree with the increase in AGE as determined by biochemical methods in experimental diabetes reported by other authors. For example, Monnier and colleagues[63] reported a twofold increase in collagen-linked chromophores in galactose-fed rats. A similar increase in pentosidine, another AGE component, was seen in the collagen of diabetic patients with renal disease.[64] Using a radioreceptor assay, Makita and colleagues[59] also reported that increases in AGE-modified proteins were detected in arterial collagen of diabetic patients, and Nakamura et al[38] found AGE in the arteries of diabetic human subjects but not in those of nondiabetic individuals.

When atherosclerotic lesions of WHHL rabbits were studied, we observed a striking coincidence of staining with antibodies directed at glycosylation end products and antibodies directed at oxidation-specific epitopes. The general distribution of immunostaining obtained

with antibodies to epitopes typically generated during oxidative modification of LDL, eg, MDA-lysine and 4-hydroxynonenal–lysine, was very similar to that obtained with AGE-specific antibodies (Fig 9). Immunocytochemistry with FFI-specific antibodies showed more extracellular, diffuse, and reticular staining in earlier lesions than with oxidation-specific antibodies and some staining of the media. Nevertheless, the similarity of the intense staining of the "shoulder" areas of lesions (rich in both macrophage/foam cells and lipids) and of the adventitia is of great interest. Cross-reactivity between the antisera to FFI and AGE and oxidation-specific antisera is most unlikely, given the very different chemical nature of the antigens used to prepare the antisera. The results of our competitive RIAs support this idea (Fig 4B). Furthermore, by including FFI-albumin and FFI-KLH among the antigens used to generate antisera against FFI-like structures, we minimized the probability of generating antibodies against lipid peroxidation epitopes. The colocalization of oxidation-specific adducts and AGE-specific epitopes within the intimal lesion could be due in part to cross-linking of lipoproteins by AGE,[15] which would prolong the half-life of LDL in the artery wall and expose it to further oxidative modification by arterial macrophages or smooth muscle cells. It is also possible that in vivo, certain immunologically related epitopes may be formed during oxidation of LDL and the oxidative reactions involved in the formation of AGE.[29,30] Perhaps more likely is the possibility that products of lipid peroxidation may enhance AGE formation, which may in turn enhance lipid per-

oxidation (reviewed in References 65 and 66). For example, products of lipid peroxidation have been reported to increase the cross-linking of nonenzymatically glycosylated collagen in vitro, and conversely, glucose and glycosylated collagen have been reported to increase free-radical production and to catalyze lipid peroxidation.[12,31-34] In addition, low-molecular-weight peptides resulting from breakdown of AGE proteins are found in the circulation and bind to LDL,[67] and conceivably these could also enhance the susceptibility of LDL to oxidation. Thus, lipid peroxidation and formation of Amadori products and AGEs may be intricately linked and could explain the apparent colocalization of the immunostaining patterns in lipid-rich atherosclerotic lesions, even in nondiabetic animals.

Although colocalization is consistent with the hypothesized mutual enhancement of oxidation and AGE formation, the increased AGE formation in atherosclerotic lesions of LDL receptor–deficient rabbits does not provide conclusive evidence, as both lipoprotein oxidation and AGE formation may independently result from increased generation of free radicals in this animal model. For example, hypercholesterolemia per se may increase endothelial generation of superoxide anion.[68]

Even though the simple covalent attachment of a sugar to autologous protein would not be expected to be immunogenic, autoantibodies that are prevalent in the sera of normal and diabetic human subjects recognize both Amadori products[69] and epitopes of AGE.[11] Autoantibodies that recognize several forms of OxLDL, in particular MDA-lysine, are prevalent in humans and
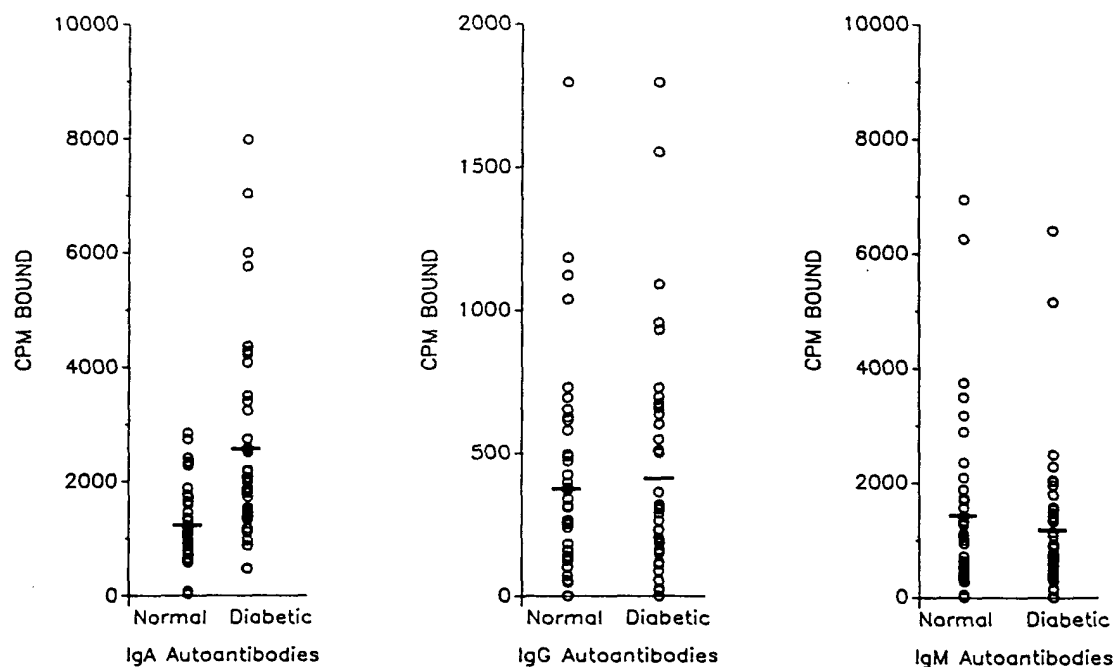

Fig 12.   Binding of IgA, IgG, and IgM autoantibodies in the sera of human diabetic and control subjects to FFI-LDL. The antigen in this solid-phase radioimmunoassay was human FFI-LDL plated at 5 μg/mL. Serum (50 μL) (1:8 dilution for determination of IgA and IgG and 1:256 dilution for IgM autoantibodies) was added as the primary antibody, and bound antibodies were detected by radiolabeled monoclonal antibodies (second antibody) against human IgA, IgG, or IgM (see "Methods"). For each sample, a control well without antigen was subtracted as background. Data are expressed as counts per minute (cpm) of the indicated bound second antibody. Mean cpm values were not different for IgG and IgM class antibodies but were significantly different for IgA (P<.001). See the legend to Fig 3 for explanation of other abbreviations.

other species.[40,49] These autoantibodies are capable of binding to epitopes of OxLDL in lesions,[48] and immunoglobulins isolated from atherosclerotic lesions, recognize OxLDL, which may be present in lesions as immune complexes with OxLDL.[70] Such autoantibodies may not only be markers of protein modification but also modulators of atherogenesis by forming immune complexes with their respective antigens in the circulation or within the arterial wall. In addition, cell-mediated immunity may also occur. Increasing evidence indeed suggests an important role for the immune system in the atherogenic process.[71,72]

In the limited study of diabetic and control sera, no differences in titers of autoantibodies against FFI-LDL of IgG and IgM were found, but diabetic subjects showed significantly higher concentrations of autoantibodies of IgA. Because titers of circulating autoantibodies reflect genetic control of the response to a given antigen as well as variations in the production and consumption of antibodies, the interpretation of antibody titers is complex. The possible clinical relevance of this observation remains unclear and requires further testing in more rigidly matched patient populations. However, the fact that circulating autoantibodies to FFI-like structures were present in the sera of both normal and diabetic subjects provides further evidence for the in vivo occurrence of FFI or immunologically similar epitopes.

Acknowledgments

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